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(54) Title: TREATMENT OF HYPERPROLIFERATIVE DISORDERS

(57) Abstract

A method of treating a hyperproliferative disorder in a subject in need of such treatment, comprising administering to said subject, in combination, a treatment effective amount of: (a) a ceramide–generating retinoid such as fenretinide or a pharmaceutically acceptable salt thereof; and (b) at least one (and in certain embodiments at least two) ceramide degredation inhibitor, such as compounds selected from the group consisting of (i) glucosylceramide synthesis inhibitors, (ii) sphingosine–1–phosphate synthesis inhibitors, and (iii) protein kinase C inhibitors. A preferred glucosyl ceramide synthesis inhibitor is 1–phenyl–2–palmitoylamino–3–morpholino–1–propanol. A preferred sphingosine–1–phosphate synthesis inhibitor is D–erytho–N,N–dimethylsphingosine. A preferred protein kinase C inhibitor is L–threo–dihydrosphingosine.

TREATMENT OF HYPERPROLIFERATIVE DISORDERS

Field of the Invention

The present invention concerns combination chemotherapy regimes for the treatment of hyperproliferative disorders, and formulations useful for carrying out the same.

Background of the Invention

Fenretinide [HPR; all-*trans*-N-(4-hydroxyphenyl)retinamide; CAS Registry number 65646-68-6] is currently believed to effect cytotoxicity in cancer cells by generating reactive oxygen species. *See, e.g.,* D. Delia et al., *Carcinogenesis* 18, 943-948 (1997); N. Oridate et al., *J. Natl. Cancer Inst.* 89, 1191-1198 (1997).

U.S. Patent No. 4,665,098 to Gibbs describes pharmaceutical compositions of fenretinide as useful for the treatment of breast and bladder cancer.

U.S. Patent No. 5,821,072 to Schwartz et al. provides methods for screening protein kinase C inhibitors capable of potentiating apoptosis in tumor cells, along with methods for screening antitumor therapeutic agents suitable for combination therapy with a protein kinase C inhibitor capable of potentiating apoptosis in tumor cells.

Summary of the Invention

The present invention is based on the unexpected discovery that fenretinide at appropriate doses generates increased and sustained ceramide in human cancer cell lines. Thus, the cytostatic or cytotoxic activity against hyperproliferative disorders

combination, a treatment effective amount of: (a) a retinoic acid derivative that generates ceramide such as fenretinide or a pharmaceutically acceptable salt thereof; and (b) a sphingosine-1-phosphate synthesis inhibitor such as D-erythro-N,N-dimethylsphingosine or a pharmaceutically acceptable salt thereof. The sphingosine-1-phosphate synthesis inhibitor is administered in an amount effective to enhance the activity of the retinoic acid derivative, such that the two compounds together have an efficacious activity. Preferably, the retinoic acid derivative is given in an amount effective to produce necrosis, apoptosis, or both in the tumor cell, and the sphingosine-1-phosphate synthesis inhibitor is given in an amount effective to increase the necrosis, apoptosis, or both produced in the tumor cell over that which would be produced by the retinoic acid derivative alone, or that expected to be produced by the sum of that produced by the retinoic acid derivative and sphingosine-1-phosphate synthesis inhibitor when given separately.

Also disclosed is a method of treating a hyperproliferative disorder in a subject in need of such treatment, the method comprising administering to the subject, in combination, a treatment effective amount of: (a) a retinoic acid derivative that generates ceramide such as fenretinide or a pharmaceutically acceptable salt thereof; and (b) a protein kinase C inhibitor such as L-threo-dihydrosphingosine or a pharmaceutically acceptable salt thereof. The protein kinase C inhibitor is administered in an amount effective to enhance the activity of the retinoic acid derivative, such that the two compounds together have an efficacious activity. Preferably, the retinoic acid derivative is given in an amount effective to produce necrosis, apoptosis or both, in the tumor cell, and the protein kinase C inhibitor is given in an amount effective to increase the necrosis, apoptosis or both produced in the tumor cell over that which would be produced by the retinoic acid derivative alone, or that expected to be produced by the sum of that produced by the retinoic acid derivative and protein kinase C inhibitor when given separately.

Also disclosed is a method of treating a hyperproliferative disorder in a subject in need of such treatment, comprising administering to said subject, in combination, a treatment effective amount of: *(a)* a ceramide-generating retinoid or a pharmaceutically acceptable salt thereof; and (b) at least two (*e.g.*, 2 or 3) compounds selected from the group consisting of *(i)* glucosylceramide synthesis inhibitors, *(ii)* sphingosine-1-phosphate synthesis inhibitors, and *(iii)* protein kinase C inhibitors. The at least two compounds are administered in an amount effective to enhance the

synthase inhibitor, on cell survival in a highly HPR resistant cell line (SK-N-RA), at varying concentrations. Filled circles represent the combination of safingol and ppmp; open circles represent the combination of fenretinide and safingol; filled triangles represent the combination of fenretinide and ppmp; open triangles represent the combination of fenretinide, safingol and ppmp. Dosages are as indicated on the horizontal axis.

Figure 5 illustrates the effect of various compound combinations with dosages varrying as indicated, but at a fixed 10 μM dose of fenretinide, on the survival of SK-N-RA cells. T or Tamoxifen refers to tamoxifen citrate. Filled circles labelled H+P represent fenretinide plus ppmp; open circles labelled H+T represent fenretinide plus tamoxifen; filled circles labelled H+S represent fenretinide plus safingol; open circles H+S+T represent fenretinide plus safingol and tamoxifen (1:1); filled trinagles represent fenretinide plus 3 μM tamoxifen fixed plus safingol. Other dosages are as indicated on the horizontal axis.

Figure 6 shows the activity of low dosage fenretinide in combination with other compounds on the survival fraction of SK-N-RA cells. Filled circles represent 3.3 μM fenretinide plus safingol; open circles represent 3.3 μM fenretinide plus PPMP; filled triangles represent PPMP plus safingol (1:1) without fenretinide; open triangles represent 3.3 μM fenretinide plus PPMP plus safingol (1:1). Other dosages are as indicated on the horizontal axis.

Figure 7 shows the effect of various drug combinations on the survival fraction of SK-N-RA cells. N-DMS (or N) refers to d-erythro-N,N-dimethylsphingosine, a sphingosine kinase inhibitor. Filled circles represent N-DMS plus ppmp; open circles represent fenretinide plus ppmp; filled triangles represent fenretinide plus N-DMS; open triangles represent fenretinide plus N-DMS plus ppmp. Dosages are as indicated on the horizontal axis.

Figure 8 illustrates the activity of various drug combinations on the survival of SK-N-RA cells. Filled circles labelled HPR represent fenretinide; open circles labelled N-DMS represent N-DMS; filled triangles represent HPR plus N-DMS; filled circles labelled 10μM H+N represent a 10 μM fixed dose of fenretinide plus N-DMS; open circles labelled 5 μM H+P+N represent 5 μM fenretinide fixed dose plus 5 μM ppmp fixed dose plus N-DMS. The solid line represents fenretinide plus ppmp.

Detailed Description of the Preferred Embodiments

The methods of the present invention utilize the combined effects of retinoic acid derivatives and an agent (*i.e.*, a potentiating agent) that manipulates cellular metabolism and cellular control of ceramide-generated toxicity, in order to inhibit or prevent the growth of tumors, cancers, neoplastic tissue and other premalignant and noneoplastic hyperproliferative disorders, all of which are together referred to as hyperproliferative or hyperplastic disorders herein. The treatments employed herein may be used to inhibit growth and/or to induce cytotoxicity (by necrotic or apoptotic mechanisms, or both) in the target cells, which are generally hyperproliferative cells (including tumors, cancers, and neoplastic tissue, along with pre-malignant and non-neoplastic or non-malignant hyperproliferative disorders).

Examples of tumors, cancers, and neoplastic tissue that can be treated by the present invention include but are not limited to malignant disorders such as breast cancers; osteosarcomas; angiosarcomas; fibrosarcomas and other sarcomas; leukemias; lymphomas; sinus tumors; ovarian, uretal, bladder, prostate and other genitourinary cancers; colon esophageal and stomach cancers and other gastrointestinal cancers; lung cancers; myelomas; pancreatic cancers; liver cancers; kidney cancers; endocrine cancers; skin cancers; and brain or central and peripheral nervous (CNS) system tumors, malignant or benign, including gliomas and neuroblastomas.

Examples of premalignant and non-neoplastic or non-malignant hyperproliferative disorders include but are not limited to myelodysplastic disorders; cervical carcinoma-in-situ; familial intestinal polyposes such as Gardner syndrome; oral leukoplakias; histiocytoses; keloids; hemangiomas; hyperproliferative arterial stenosis, inflammatory arthritis; hyperkeratoses and papulosquamous eruptions including arthritis. Also included are viral induced hyperproliferative diseases such as warts and EBV induced disease (*i.e.*, infectious mononucleosis), scar formation, and the like. The methods of treatment disclosed herein may be employed with any subject known or suspected of carrying or at risk of developing a hyperproliferative disorder as defined herein.

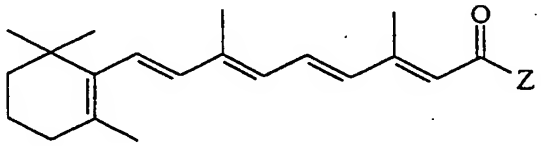
As used herein, "treatment" of a hyperproliferative disorder refers to methods of killing, inhibiting or slowing the growth or increase in size of a body or population of hyperproliferative cells or tumor or cancerous growth, reducing hyperproliferative

proteins), with caspase activation being a late step in the final events leading to apoptotic cell death. However, not all cell death occurs via apoptosis, and cell death induced by 4-HPR involves both apoptosis and necrosis (J. Clifford et al., *Cancer Res.* 59, 14 (1999)). The intracellular lipid ceramide is known to mediate apoptosis (L. Obeid et al., *Science* 259, 1769 (1993)(Figure 1) and necrosis (Guo et al., *Am. J. Physiol.* **276**, F390 (1999); Condorelli et al., *Br. J. Pharmacol.* **137**, 75 (1999)). It has been shown to cause the apoptosis-inducing permeability transition of mitochondrial membranes (S. Susin et al., *J. Exp. Med.* 186, 25 (1997)), cause apoptosis-inducing ROS generation by mitochondrial complex III inhibition (A. Quillet-Mary et al., *J. Biol. Chem.* 272, 21388 (1997) and activate the pro-death JNK/SAPK pathway (S. Basu et al., *Oncogene* 17, 3277 (1998); T. Okazaki et al., *Cell. Signal.* 10, 685 (1998); W. Jarvis, *Curr. Opin. Oncol.* 10, 552 (1998)). Ceramide also activates a protein kinase (CAPK) (S. Mathias et al., *Biochem. J.* 335(Pt 3), 465 (1998) and a phosphorylase (PP2A) (L. Leoni et al., *Biochem. Pharmacol.* 55, 1105 (1998)) and can lead to the activation of the nuclear transcription factor, NF-kappaB (L. Johns et al., *J. Immunol.* 152, 5877 (1998); C. Gamard et al., *J. Biol. Chem.* 272, 1682 (1997)). Mechanisms by which cancer cells avoid the cytotoxic effects of ceramide can include metabolism to other forms, including nontoxic glucosylceramide (Y. Lavie et al., *J. Biol. Chem.* 272, 1682 (1997); Y. Lavie et al., *J. Biol. Chem.* 271, 19530 (1996); L. Yong-Yu et al., *J. Biol. Chem.* 274, 1140 (1999)) and sphingosine-1-phosphate. Sphingosine-1-phosphate opposes ceramide-induced cell death by activating the pro-life ERK1/2 pathway (O. Cuvillier et al., *Nature* 381, 800 (1996); O. Cuvillier et al., *J. Biol. Chem.* 273, 2910 (1998)). Thus, modulation of ceramide metabolism offers a means for enhancing the cytotoxic efficacy of 4-HPR (fenretinide) and other ceramide-generating retinoids.

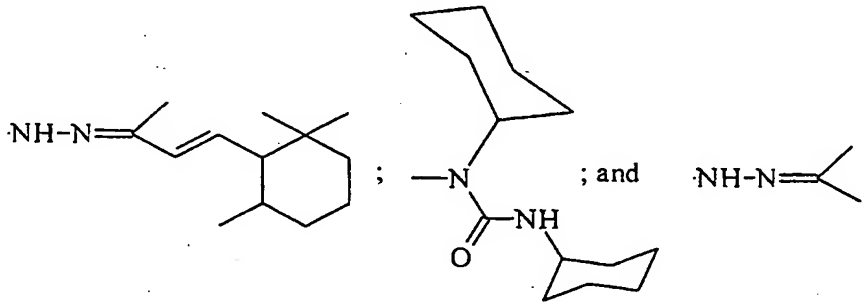
Some of the key metabolic pathways involved in the synthesis and metabolism of ceramide.are shown in Figure 2. (Y. Hannun, *Science* 274, 1855 (1996). Ceramide is generated intracellularly via activation of (1) ceramide synthase, the *de novo* synthetic pathway or by activation of the (2) neutral- or acidic- sphingomyelinases, leading to breakdown of sphingomyelin. Ceramide is metabolized to (3) non-cytotoxic glucosylceramide by glucosylceramide synthase; and converted into (4) cytotoxic sphingosine by alkaline- or acidic- ceramidases. Sphingosine is further converted to the anti-apoptotic (5) sphingosine-1-phosphate by sphingosine kinase. We show below that modulation of these pathways can enhance, even synergistically

wherein Y is a member selected from the group consisting of: cholesteryloxy; phenyl; 4-bromophenyl; 4-methoxyphenyl; 4-nitrophenyl; 4-hydroxyphenyl; 4-methylphenyl; 4-cyanophenyl; 4-ethoxyphenyl; 4-acetoxyphenyl; 2-naphthyl; 4-biphenyl; 2,5-dimethoxyphenyl; 2,4-dichlorophenyl; 2,4-dimethylphenyl; 3,4-diacetoxyphenyl; 3,4,5-trimethoxyphenyl; and 2,4,6-trimethylphenyl; and

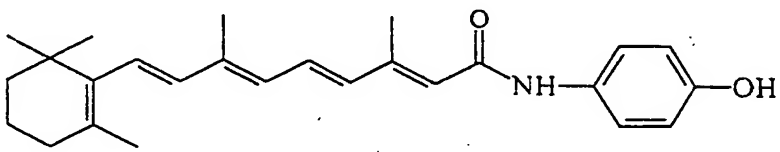
(C) amides of all-trans-retinoic acid having the following formula:



wherein Z is a member selected from the group consisting of: n-propylamino; tert-butylamino;1,1,3,3-tetramethylbutylamino; 1-morpholino; 4-hydroxyphenylamino; 4-carbomethoxy-2-hydroxyphenylamino; beta-(3,4-dimethoxyphenyl)-ethylamino; 2-benzothiazolylamino; 1-imidazolyl; 1-(2-nicotinoylhydrazolyl); 1-benzotriazolyl; 1-(1,2,4-triazolyl);



Particularly preferred is all-*trans*-N-(4-hydroxyphenyl)retinamide, also called fenretinide, which has CAS registry number 65646-68-6, and has the structure:



The foregoing compounds can be prepared in accordance with known techniques. *See, e.g.*, U.S. Patent No. 4,190,594 to Gander et al.; U.S. Patent No. 4,665,098 to Gibbs.

and the D enantiomer of such compounds are preferred. Such compounds are known and are disclosed, for example, in U.S. Patent No. 5,302,609 to Shayman and Radin: U.S. Patent No. 5,041,441 to Radin et al.; and U.S. Patent No. 5,707,649 to Inokuchi et al. Specific examples of glucosylceramide synthase inhibitors include:

1-phenyl-2-acylamino-3-morpholino-1-propanol in which n is 6 to 12;

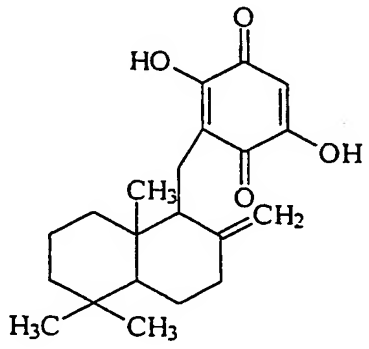
1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP);

1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP); and

Tamoxifen, including tamoxifen citrate.

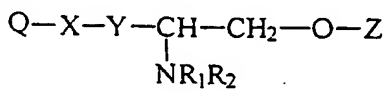
3. Sphingosine-1-phosphate synthesis inhibitors.

Any sphingosine-1-phosphate synthesis inhibitor can be used to carry out the present invention, with sphingosine kinase inhibitors such as D-erythro-N,N-dimethylsphingosine currently preferred. Additional sphingosine kinase inhibitors are known. For example, the compound may be Sankyo Co. sphingosine kinase inhibitor F12509A (or a pharmaceutically acceptable salt thereof), disclosed in Japanese Patent Application 9176083 (1997) and having the structure:



4. Protein kinase C inhibitors.

Example protein kinase C inhibitors include those described in U.S. Patent No. 4,816,450 to Bell et al. Such compounds include those having the general formula:



wherein Q is CH_3-$(CH_2)_n$- or CH_3-$(CH_2)_m$-CH=CH-$(CH_2)_p$- wherein n is 2-30, m is 1-15 and p is 1-15;

In active compounds that interact with a receptor, the interaction takes place at the surface-accessible sites in a stable three-dimensional molecule. By arranging the critical binding site residues in an appropriate conformation, compounds which mimic the essential surface features of the active compound binding region may be designed and synthesized in accordance with known techniques. A molecule which has a surface region with essentially the same molecular topology to the binding surface of the active compound will be able to mimic the interaction of the active compound with its corresponding receptor. Methods for determining the three-dimensional structure of active compounds and producing active analogs thereof are known, and are referred to as rational drug design techniques. *See, e.g.,* U.S. Patent No. 5,593,853 to Chen; U.S. Patents Nos. 5,612,895 and 5,331,573 to Balaji et al.; U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; and U.S. Patent No. 4,863,857 to Blalock (the disclosures of all U.S. Patent references cited herein are to be incorporated herein by reference).

In combinatorial chemistry (or random drug design) techniques, large combinatorial libraries of candidate compounds are screened for active compounds therein. Libraries used to carry out the present invention may be produced by any of a variety of split synthesis methods. Split synthesis methods in which a releasable tag is attached to the particle along with the organic compounds of interest are also known as cosynthesis methods. A variety of such methods are known. *See, e.g.,* A. Furka et al., *J. Pept. Protein Res. 37,* 487 (1991); K. Lam et al., *Nature* **354,** 82 (1991); R. Zuckermann et al., *Int. J. pept. Protein Res.* **40,** 498 (1992); F. Sebestyen et al., *Bioorg. Med. Chem. Lett.* 3, 413 (1993); K. Lam et al., *Bioorg. Med. Chem. Lett.* 3, 419 (1993). For example, the library may be a library of organometallic compounds wherein the compound is a metal-ligand complex. The metal in the complex may be an early or late transition metal in high, low or zero oxidation states. The metal may also be any of the main group metals, alkali metals, alkaline earths, lanthanides or actinides. The ligand in the metal-ligand complex may be composed of, or derived from, chiral or achiral forms of cyclopentadienes, amino esters, oxazolidoinones, hydroxy acids, hydroxy esters, hydroxy amides, pyridines, fused pyridines, nitrogen heterocycles, oxazoles, imidazoles, pyrroles, crown ethers, cryptands, carcerands, phosphines, diphosphines, polyphosphines, quinuclidines, quinines, alkaloids, dextrins, cyclodextrins, salens. porpyrins. biaryls, sulfonamides,

(c) contacting experimental tumor cells with both said amount of ceramide generating retinoid in step (a) above and said amount of a test compound in step (b) above; and

(d) determining the growth inhibition of said tumor cells of steps (a), (b) and (c) above; and then

(e) comparing the growth inhibition or cytotoxic activity in the experimental tumor cells of step (c) with the growth inhibition of the control tumor cells of steps (a) and (b), a greater degree of growth inhibition determined in the experimental tumor cells of step (c) than the combined growth inhibition of the control tumor cells of steps (b) and (c) indicating that the test compound enhances the activity of the ceramide-generating retinoid.

The comparing step may be carried out by any suitable means, such as by calculating a Combination Index, where a value less than 1 (e.g., less than 0.9) indicates the compounds are synergistic. Any tumor cells can be used, including but not limited to neuroblastoma, lung, melanoma, prostate, leukemia, colon, breast, and pancreas tumor cells. Any ceramide-generating retinoid such as fenretinide can be used. Other hyperproliferative cells including pre-malignant and non-malignant cells can be used instead of tumor cells, as noted with respect to conditions for treatment above. In preferred embodiments, the test compound is a ceramide-degradation inhibitor, or other agent that manipulates cellular metabolism or cellular control of ceramide-generated cytotoxicity. The determining step may be carried out by looking for growth inhibition or cytotoxicity in general, or by particularly determining necrosis, apoptosis, or both. The method may be used to identify active compounds that are ceramide-degradation inhibitors, other compounds that manipulate cellular metabolism or cellular control of ceramide-generated cytotoxicity, or compounds that operate by still other mechanisms in addition to those described herein.

Compounds (including the pharmaceutically acceptable salts thereof) that have not previously been known as useful in a method of treating hyperproliferative diseases in combination with a ceramide-generating retinoid, can be prepared, formulated and used in the methods described herein in addition to, or in alternative to, the ceramide-degradation inhibitors described herein. Depending upon the compounds selected for screening, such compounds may be novel compounds, may be known compounds but not previously known for a medicinal or pharmaceutical use, may be compounds previously known for a medicinal or pharmaceutical use but

or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the active compound, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the compound in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

Formulations suitable for buccal (sub-lingual) administration include lozenges comprising the active compound in a flavoured base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Formulations of the present invention suitable for parenteral or vaginal administration conveniently comprise sterile aqueous preparations of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may be administered by means of subcutaneous, intravenous, intramuscular, or intradermal injection. Such preparations may conveniently be prepared by admixing the compound with water or a glycine buffer and rendering the resulting solution sterile and isotonic with the blood.

Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These may be prepared by admixing the active compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include vaseline, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration may also be delivered by iontophoresis (*see*, for example, *Pharmaceutical Research* 3 (**6**):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound. Suitable formulations

fluorescence of a drug-treated cell population to the fluorescence of a similar number of untreated cells yields a survival fraction. In brief, 5000 to 10.000 SK-N-RA neuroblastoma cells/well are replicate plated into 60 wells of a 96-well tissue culture plate in 0.1 cc media and allowed to attach overnight. Drug(s) are then added in 0.05 cc media to the final concentrations indicated. There are 12 wells treated per drug concentration. Twelve wells receive drug-vector only to the appropriate final concentration and serve as controls for the plate. Cells are incubated for 96-120 hours at 37°C in 5% CO_2. Fluorescein diacetate is then added to each well in 0.05 cc media to a final concentration of 8 microgram/cc. Cells are incubated for a further 15 minutes at 37°C and 0.03 cc of 0.5% eosin Y is added to each well. Total fluorescence of viable cells is then measured by digital imaging microscopy.

EXAMPLE 2

Ceramide Assay

The ceramide assay is carried out as follows. 500,000 neuroblastoma cells/well are replicate plated in six-well tissue culture plates and allowed to attach overnight. Tritiated (3H)-palmitic acid (a lipid precursor) is added to 1 microcure/cc and fenretinide added to a final concentration of 10 uM. Control cells receive tritiated label but no drug. At the indicated time, cells are harvested from triplicate wells, washed, and lipids extracted with methanol, acetic acid, water, and chloroform. The organic layer (containing the tritium label incorporated into lipids) is isolated and dried down by a nitrogen stream. The lipid sample is dissolved in chloroform: methanol and 10% of each sample assayed to estimate the total tritium in the lipid sample. The lipids in sample fractions, together with unlabeled ceramide standards, are then separated by thin layer chromatography and the plates developed by iodine vapor. The region of the plate corresponding to the ceramide standard is scraped and the tritium of the co-migrating sample ceramide is measured. Total sample ceramide is then expressed as percent tritium label in ceramide versus tritium in total lipid.

open triangles represent 3.3 μM fenretinide plus ppmp plus safingol (1:1). Other dosages are as indicated on the horizontal axis

Figure 7 shows the effect of various drug combinations on the survival fraction of sk-N-RA cells at 20% O_2. N-DMS (or N) refers to d-erythro-N,N-dimethylsphingosine, a sphingosine kinase inhibitor. Filled circles represent N-DMS plus PPMP; open circles represent fenretinide plus ppmp; filled triangles represent fenretinide plus N-DMS; open triangles represent fenretinide plus N-DMS plus PPMP. Dosages are as indicated on the horizontal axis.

Figure 8 illustrates the activity of various drug combinations on the survival of sk-N-RA cells at 20% O_2. Filled circles labelled HPR represent fenretinide; open circles labelled N-DMS represent N-DMS; filled triangles represent HPR plus N-DMS; filled circles labelled 10 μM H+N represent a 10 μM fixed dose of fenretinide plus N-DMS; open circles labelled 5 μM H+P+N represent 5 μM fenretinide fixed dose plus 5 μM PPMP fixed dose plus N-DMS. the solid line represents fenretinide plus PPMP. Dosages are fixed where so indicated; otherwise dosages are as shown on the horizontal axis. Note the increased cytotoxicity when N-DMS is added to fenretinide and PPMP.

Figure 9 illustrates the activity of drug combinations on the survival of sk-N-RA cells at 20% O_2. Filled circles represent fenretinide; open circles represent fenretinide plus N-DMS (3:1); filled triangles represent fenretinide plus safingol (3:1); open triangles represent fenretinide plus N-DMS plus safingol (3:1:1). Dosages are as indicated on the horizontal axis. Note the cytotoxicity of the three drug combination.

EXAMPLE 10

All Compounds Need Not be Co-Present for the Entire Treatment Period

In some cell lines, we have demonstrated that safingol need only be co-present with HPR for a portion of the entire treatment period in order to obtain an increase in anti-tumor cell activity. In these experiments, safingol and HPR were added together at Time = 0. Then, at various times, the cell culture medium containing both drugs was removed and replaced with medium that contained a similar concentration of HPR-only. Cells were then allowed to complete 96-120 hour incubations and their survival compared to cells that had been exposed to both drugs for the entire 96-120

invention to function. This demonstrates that all compounds need not be present at all times for the invention to function.

EXAMPLE 11

The cytotoxicity of other retinoids is increased by safingol

The retinoid, all-trans-retinoic acid (ATRA), has been previously shown to cause modest (1.5x) increases in the level of ceramide of Neuro2a neuroblastoma cells (L. Riboni et al., *J. Biol. Chem.* **270**: 26868 (1995)). Here we demonstrate that the co-exposure of ATRA, or the retinoid, 13-cis-retinoic acid, with safingol results in significantly decreased cell survival in CHLA-90 and LAN-6 neuroblastoma cells compared to that of either retinoid alone. This demonstrates that the invention is active with various different retinoids.

Methods. Cells were added in 100 μL whole medium per well to 96-well microplates for DIMSCAN cytotoxicityassay as previously described. Cell lines used were the neuroblastoma cell lines CHLA-90 and LAN-6. At Time = 0, either all-trans-retinoic acid (ATRA), 13-cis-retinoic acid (13-cis-RA)or a combination of retinoid plus safingol at a 3:1 molar ratio were added in 50 μL whole medium. Plates were incubated and assayed for cytotoxicity by DIMSCAN assay at +120 for CHLA-90 cells and + 144 hours for LAN-6 cells.

Results. The data set forth in **Figures 13-14** demonstrate that the addition of safingol to the retinoids ATRA or 13-cis-RA causes a significant decrease in the cell survival of the CHLA-90 and LAN-6 cell lines. Safingol at 4 μM (the maximum concentration used in the experiments below) had a Survival Fraction of 0.11in CHLA-90 and of 0.39 in LAN-6 cells. This demonstrates that the invention is active with a number of different retinoids.

EXAMPLE 12

Specific Conversion of Ceramide into Nontoxic Glucosyl-ceramide Decreases the Cytotoxicity of HPR and HPR+Safingol

We have shown that HPR generates ceramide in neuroblastoma tumor cell lines in a dose- and time- depenent manner (B. Maurer et al., *J. Natl. Cancer Inst.* (1999)(in press)). Glucosylceramide (GC) is a nontoxic metabolite of ceramide. Ceramide is converted into glucosylceramide by the action of glucosylceramide

combination (part of the Invention) is also at least partially dependent upon the generation of cytotoxic ceramide and enhancement of its cytotoxicity.

EXAMPLE 13

HPR and HPR+safingol induce cell death by a combination of apoptosis and necrosis; HPR and HPR+safingol can induce cell death by necrosis if apoptotic cell death is inhibited

There are two main mechanisms currently recognized that lead to cell death after biochemical cellular insult, *apoptosis* and *necrosis* (G. Nunez G. et al., *Oncogene* 17:3237-45 (1998); G. Cohen, *Biochem. J.* 326:1-16 (1997); Y. Hannun, *Blood* 89:1845-53 (1997); N. Thornberry, *Chem. Biol.* 5: R97-103 (1998); N. Zamzami et al., *J. Bioenerg Biomembr.* 29:185-193 (1997); D. McConkey, *Toxicol. Lett.* 99:157-98 (1998); M. Raffray and G. Cohen, *Pharmacol Ther.* 75:153-77 (1997); J. Lemasters, *Am. J. Physiol.* 276:G1-6 (1999)). Apoptosis consists of a series of fairly specific, fairly sequential, enzymatic activation steps (the caspase enzyme cascade) which usually lead to a specific type of DNA degradation (internucleosomal DNA laddering) and cell death. Apoptosis is typified morphologically by condensed nuclear chromatin and fragmentation of the nuclei into apoptotic bodies in cells which have not lost membrane integrity and an increase in sub G_0/G_1 DNA content by flow cytometry. Necrosis is a less biochemically-defined condition that is characterized by a general breakdown in cell membrane integrity and associated with decreased levels of intracellular of ATP (C. Renvoize et al., *Cell Biol. Toxicol* 14:111-20 (1998).). Necrosis is typified morphologically by a loss of membrane integrity (demonstrated by propidium iodide staining) with cell rounding and cell detachment . These two processes may overlap in parts of their biochemical mechanism, but are generally considered distinct or, at least, at opposite ends of a mechanistic continuum. As shown below, both HPR and HPR+safingol cause cell death through a combination of both apoptosis and necrosis. These observations are significant because tumor cells with impaired apoptotic mechanisms may be killed by necrosis. Thus, the drug combinations described herein have a significant advantage (induction of cell death by necrosis, as well as by apoptosis) over other methods of antitumor killing which rely primarily on an intact apoptotic mechanisms or upon enhancement of apoptosis.

Results. As shown in **Figure 16**, the cytotoxicity of HPR was significantly reduced by the pan-caspase enzyme, apoptosis-inhibitor, BOC-d-fmk (40 μM), across all HPR concentrations ($P < .001$), but HPR still induced significant cytotoxicity in the presence of BOC-d-fmk (at 3 μM HPR, $P = .002$, at > 3 μM HPR, $P < .001$). These results indicate that HPR kills cells by both apoptotic and nonapoptotic (necrotic) mechanisms.

Figure 17. Pretreatment with BOC-d-fmk prior to HPR exposure significantly reduced ($P = .001$) the morphological nuclear changes indicative of apoptosis (condensed, intensely-staining nuclear chromatin and fragmentation of the nuclei into apoptotic bodies which have not lost membrane activity) in CHLA-90 cells. However, the significant morphological evidence of necrosis ($P = .002$) induced by HPR (loss of membrane integrity demonstrated by propidium iodide staining and cell rounding) was minimally affected by BOC-d-fmk and was still significant ($P = .016$) relative to controls. HPR alone induced significant apoptosis ($P = .006$), while apoptosis in cells treated with HPR+BOC-d-fmk was not significantly different from controls ($P = .48$). These results indicate that HPR-induced cell death proceeds by mixed apoptosis/necrosis and can proceed by necrotic mechanisms even if death by apoptosis is inhibited.

Figure 18. At +24 hours, BOC-d-fmk (40 μM) abrogated the sub G_0/G_1 DNA-fragmentation induced by HPR (10 μM) in CHLA-90 as detected by flow cytometry that is a characteristic of apoptosis. As a significant fraction of CHLA-90 cells were dead or dying at +24 hours, this data provides evidence that HPR can kill cells by nonapoptotic (necrotic) mechanisms.

Cell death induced by HPR (10 –20 μM) has also recently been reported to proceed by necrosis in lymphoblastoid cell lines (L. Spreinger and B. Stewart, *Cancer Lett.* **128**:189-196 (1998)) and in an embryonal carcinoma cell line (J. Clifford et al.,*Cancer Res.* 59:14-18 (1999).).

Figure 19. Pretreatment with BOC-d-fmk prior to HPR or HPR+safingol (10:3 micromolar ratio) exposure reduced the morphological nuclear changes indicative of apoptosis in CHLA-90 cells at +48 hours. However, the morphological evidence of necrosis induced by the HPR+safingol drug combination at +48 hours was minimally affected by BOC-d-fmk and was still significant ($P <.001$) relative to controls. These results indicate that the drug combination HPR+safingol (an

TABLE 1

Combination Index of HPR + Safingol (3:1) from 0 - 12 μM HPR

Cell Type		Combination Index (CI) ED99	Log Cell Kill HPR (3:1) 9 μM	12 μM
Neuroblastoma				
SK-N-RA		<0.1	1.9	3
SMS-LHN	Pd-IND	<0.1	3.5	
CHLA-90	Pd-BMT	<0.1	3.1	4
CHLA-171		<0.1	2.9	4
CHLA-79	Pd-BMT	<0.1	3.5	4
Lung				
NCI-H146 SCLC	>c-myc	<0.1	3.2	4
NCI-H157 squamous		<0.1	2.9	4
NCI-H1792		<0.1	2.1	4
A549	p53 wt	0.2	1	2
Melanoma				
A 375	p53 wt	<0.1	2.6	4
A2058		0.2	0.9	2.7
Prostate				
LNCaP.FGC	p53 wt	<0.1	2.8	4
PC-3	p53 null	1.0	1.4	1.9
Colon				
LoVo	p53 wt	0.1	0.5	1.8
HT-29	p53 mut	0.3	1.3	2.4
Breast				
MCF7	p53 wt	0.5	1.1	1.5
DoxR MCF7		0.1	0.9	1.5
MDA-MB-231	p53 mut	0.3	0.9	3
Pancreas				
PANC-1 epithelioid	p53 mut	0.2	0.3	1.7
Hs 766T		1.7	2.9	2.5

Combination Index (CI)	Description
<0.1	very strong synergism
0.1 -0.3	strong synergism
0.3 - 0.7	synergism
0.7 - 0.85	moderate synergism
0.85 - 0.9	slight synergism
0.9 - 1.10	nearly additive
1.1 - 1.20	slight antagonism

We Claim:

1. A method of treating a hyperproliferative disorder in a subject in need of such treatment, comprising administering to said subject, in combination, a treatment effective amount of:

(a) a ceramide-generating retinoid or a pharmaceutically acceptable salt thereof; and

(b) a ceramide degradation inhibitor or a pharmaceutically acceptable salt thereof.

2. A method according to claim 1, wherein said ceramide degradation inhibitor is selected from the group consisting of glucosyl ceramide synthase inhibitors, sphingosine-1-phosphate synthesis inhibitors, protein kinase C inhibitors, and the pharmaceutically acceptable salts thereof.

3. A method according to claim 1, wherein said hyperproliferative disorder is selected from the group consisting of malignant, pre-malignant, and non-malignant hyperproliferative disorders.

4. A method of treating a hyperproliferative disorder in a subject in need of such treatment, comprising administering to said subject, in combination, a treatment effective amount of:

(a) a ceramide-generating retinoid or a pharmaceutically acceptable salt thereof; and

(b) a glucosylceramide synthesis inhibitor or a pharmaceutically acceptable salt thereof.

5. A method according to claim 4, wherein said ceramide-generating retinoid is fenretinide or a pharmaceutically acceptable salt thereof.

6. A method according to claim 4, wherein said glucosylceramide synthesis inhibitor is a glucosylceramide synthase inhibitor or a pharmaceutically acceptable salt thereof.

14. A method according to claim 12, wherein said protein kinase C inhibitor is L-threo-dihydrosphingosine or a pharmaceutically acceptable salt thereof.

15. A method of treating a hyperproliferative disorder in a subject in need of such treatment, comprising administering to said subject, in combination, a treatment effective amount of:

(a) a ceramide-generating retinoid or a pharmaceutically acceptable salt thereof; and

(b) at least two compounds selected from the group consisting of *(i)* glucosylceramide synthesis inhibitors and the pharmaceutically acceptable salts thereof, *(ii)* sphingosine-1-phosphate synthesis inhibitors and the pharmaceutically acceptable salts thereof, and *(iii)* protein kinase C inhibitors and the pharmaceutically acceptable salts thereof.

16. A method according to claim 15, wherein said ceramide generating retinoid is fenretinide or a pharmaceutically acceptable salt thereof.

17. A method according to claim 15, wherein said at least two compounds comprise *(i)* a glucosylceramide synthesis inhibitor or a pharmaceutically acceptable salt thereof and *(ii)* either a sphingosine-1-phosphate synthesis inhibitor, a protein kinase C inhibitor, or a pharmaceutically acceptable salt thereof.

18. A method according to claim 15, wherein said at least two compounds comprise a glucosylceramide synthesis inhibitor or a pharmaceutically acceptable salt thereof, and a sphingosine-1-phosphate synthesis inhibitor or a pharmaceutically acceptable salt thereof.

19. A method according to claim 15, wherein said at least two compounds comprise a glucosylceramide synthesis inhibitor or a pharmaceutically acceptable salt thereof, and a protein kinase C inhibitor or a pharmaceutically acceptable salt thereof.

20. A method according to claim 15, wherein said at least two compounds comprise a sphingosine-1-phosphate synthesis inhibitor or a pharmaceutically

25. A method according to claim 23, wherein said tumor cells are selected from the group consisting of neuroblastoma, lung, melanoma, prostate, colon, breast, leukemia and pancreas tumor cells.

26. A method according to claim 22, wherein said ceramide-generating retinoid is fenretinide or a pharmaceutically acceptable salt thereof.

27. A method according to claim 22, wherein said test compound is a ceramide-degradation inhibitor or a pharmaceutically acceptable salt thereof.

28. A method according to claim 22, wherein said step of determining growth inhibition is carried out by determining necrosis, apoptosis, or both.

29. A compound that increases the cytostatic or cytotoxic activity of a ceramide-generating retinoid in hyperproliferative cells produced by the method of claim 22, or a pharmaceutically acceptable salt thereof.

30. A pharmaceutical formulation comprising, in a pharmaceutically acceptable carrier, a treatment effective amount of a compound that increases the cytostatic or cytotoxic activity of a ceramide-generating retinoid in hyperproliferative cells produced by the method of claim 22, or a pharmaceutically acceptable salt thereof.

31. A pharmaceutical formulation according to claim 31, further comprising a treatment effective amount of a ceramide generating retinoid or a pharmaceutically acceptable salt thereof.

FIG. 1

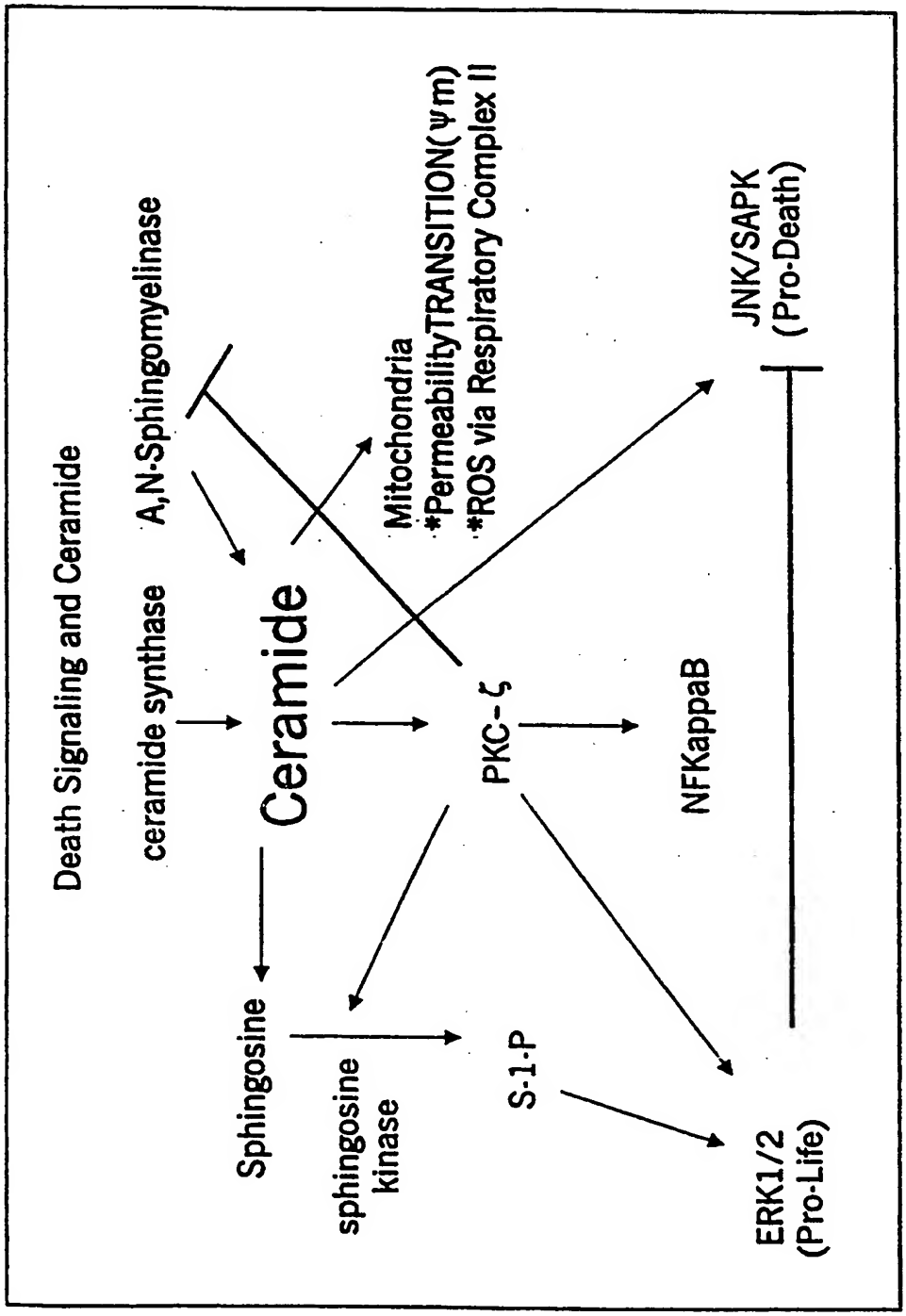

Death Signaling and Ceramide
ceramide synthase
A,N-Sphingomyelinase
Ceramide
Mitochondria
*Permeability TRANSITION(ψm)
*ROS via Respiratory Complex II
JNK/SAPK
(Pro-Death)
PKC-ζ
NFKappaB
Sphingosine
sphingosine
kinase
S-1-P
ERK1/2
(Pro-Life)

FIG. 2

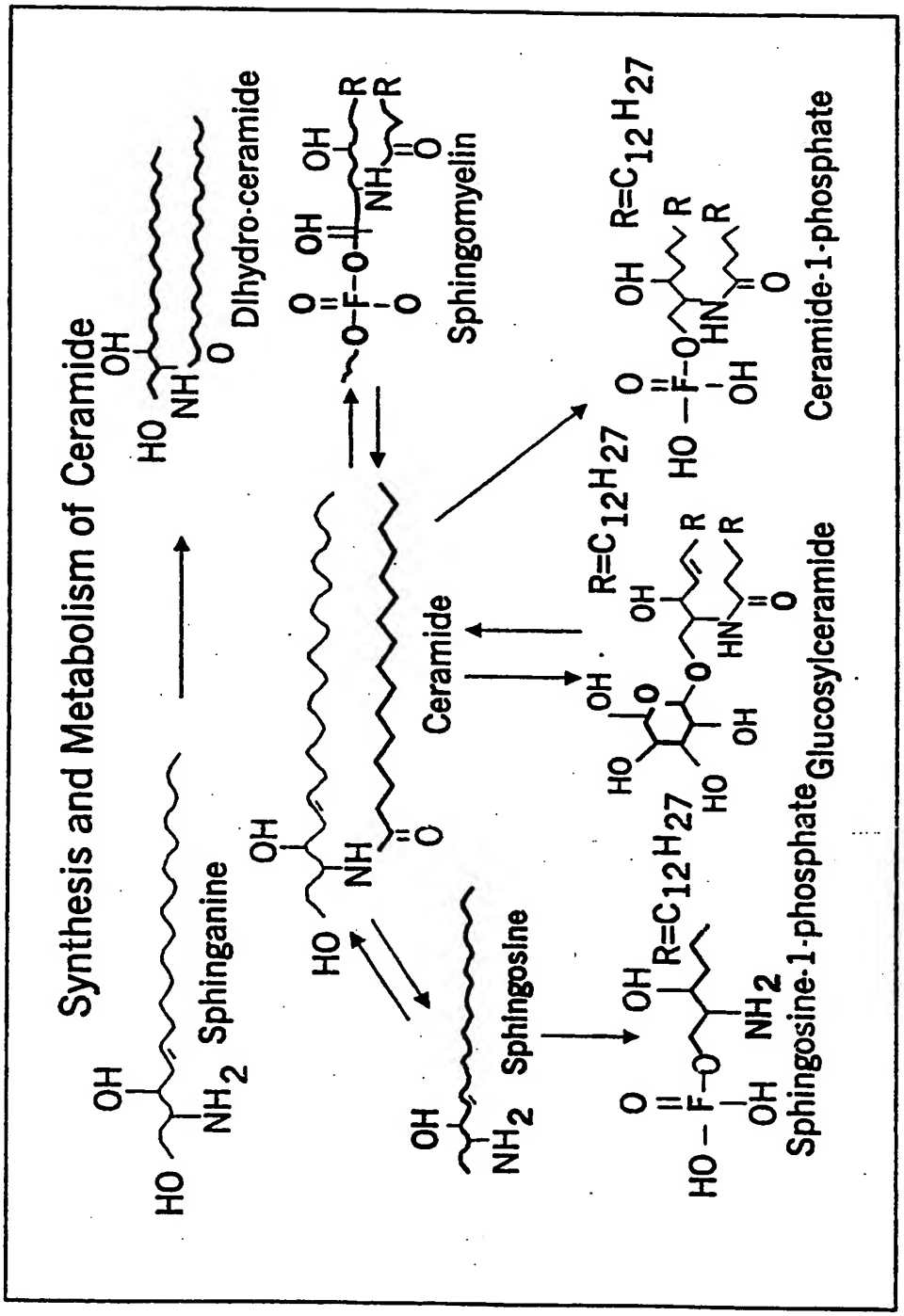
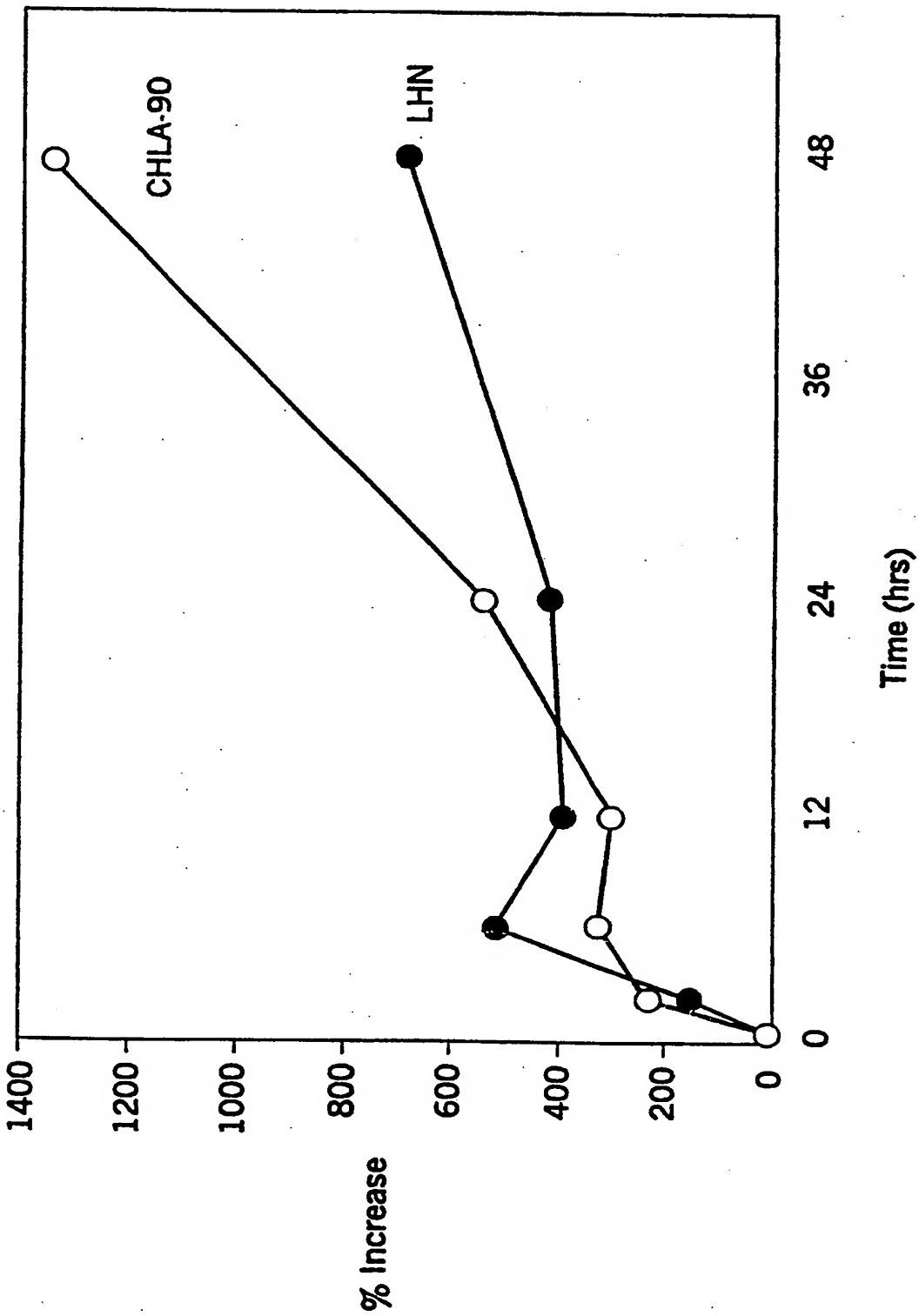

Synthesis and Metabolism of Ceramide
Sphinganine
Dihydro-ceramide
Sphingomyelin
Ceramide
Sphingosine
Glucosylceramide
Ceramide-1-phosphate
Sphingosine-1-phosphate

FIG. 3


CHLA-90
LHN
1400
1200
1000
800
600
400
200
0
% Increase
0
12
24
36
48
Time (hrs)

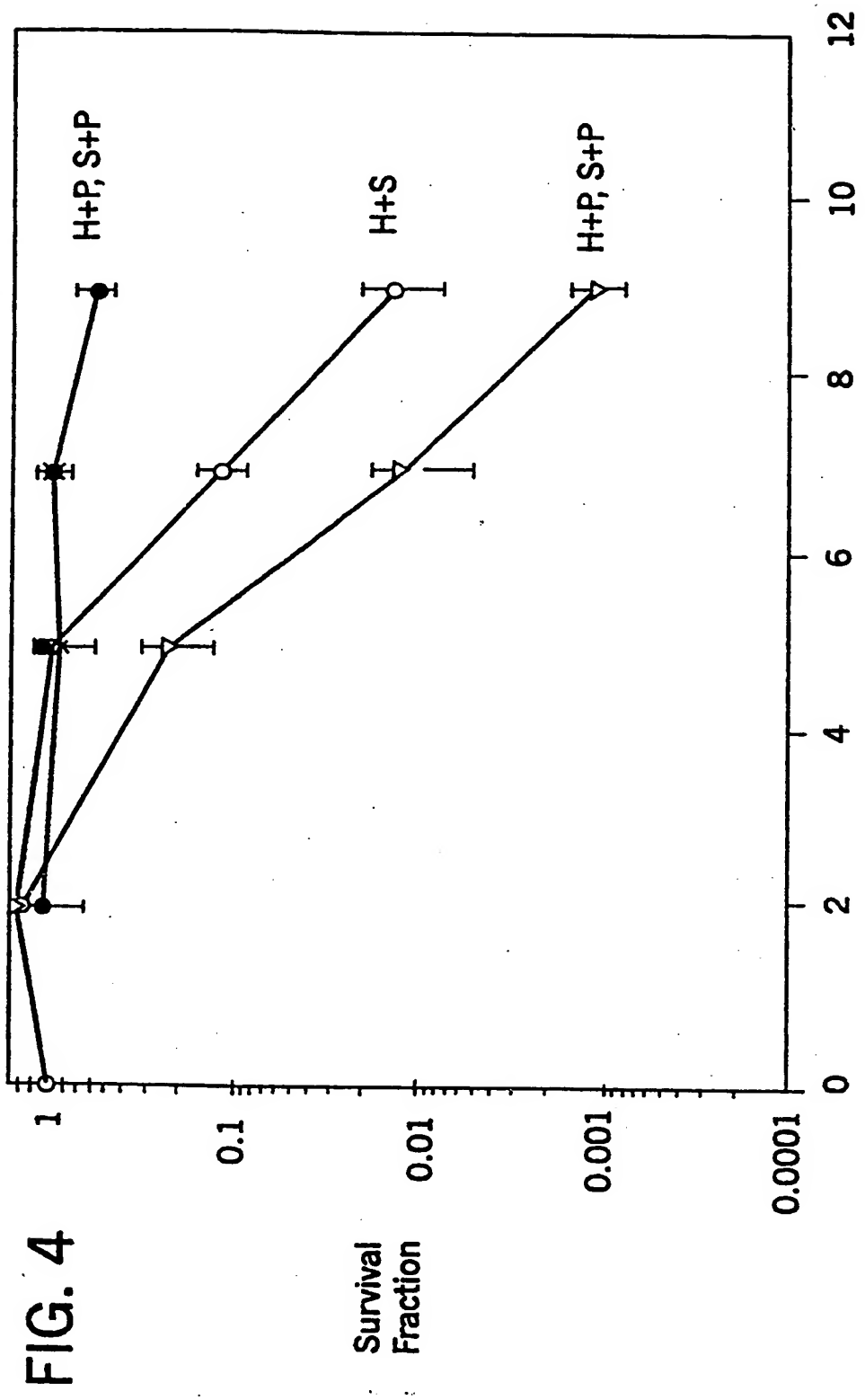

H+P, S+P
H+S
H+P, S+P
1
0.1
0.01
0.001
0.0001
Survival Fraction
0
2
4
6
8
10
12
[drug, μM]
HPR x 1
safingol x 3
ppmp x 3

FIG. 4

FIG. 5

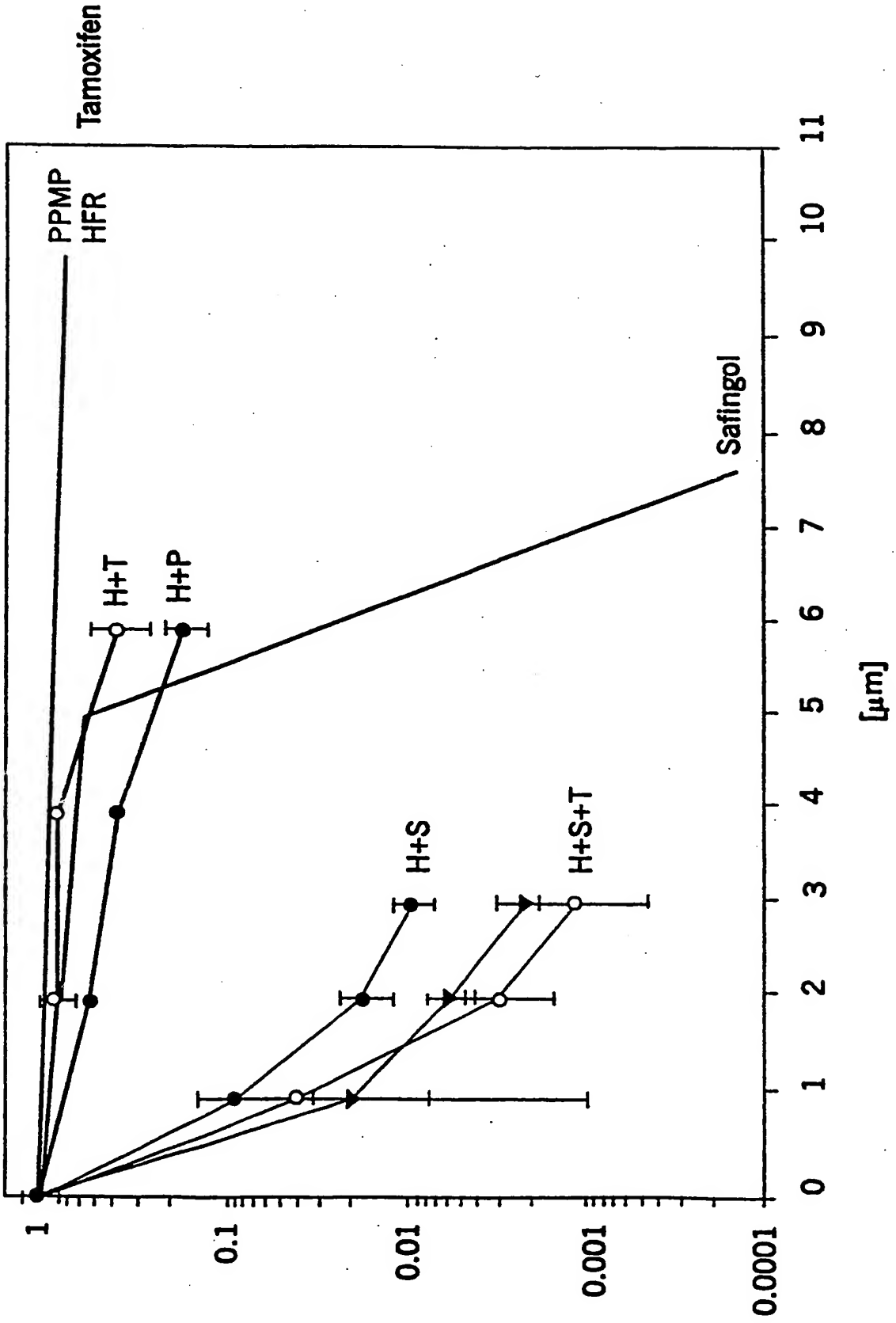

Tamoxifen
PPMP
HFR
H+T
H+P
Safingol
H+S
H+S+T
[μm]
0
1
2
3
4
5
6
7
8
9
10
11
1
0.1
0.01
0.001
0.0001

FIG. 6

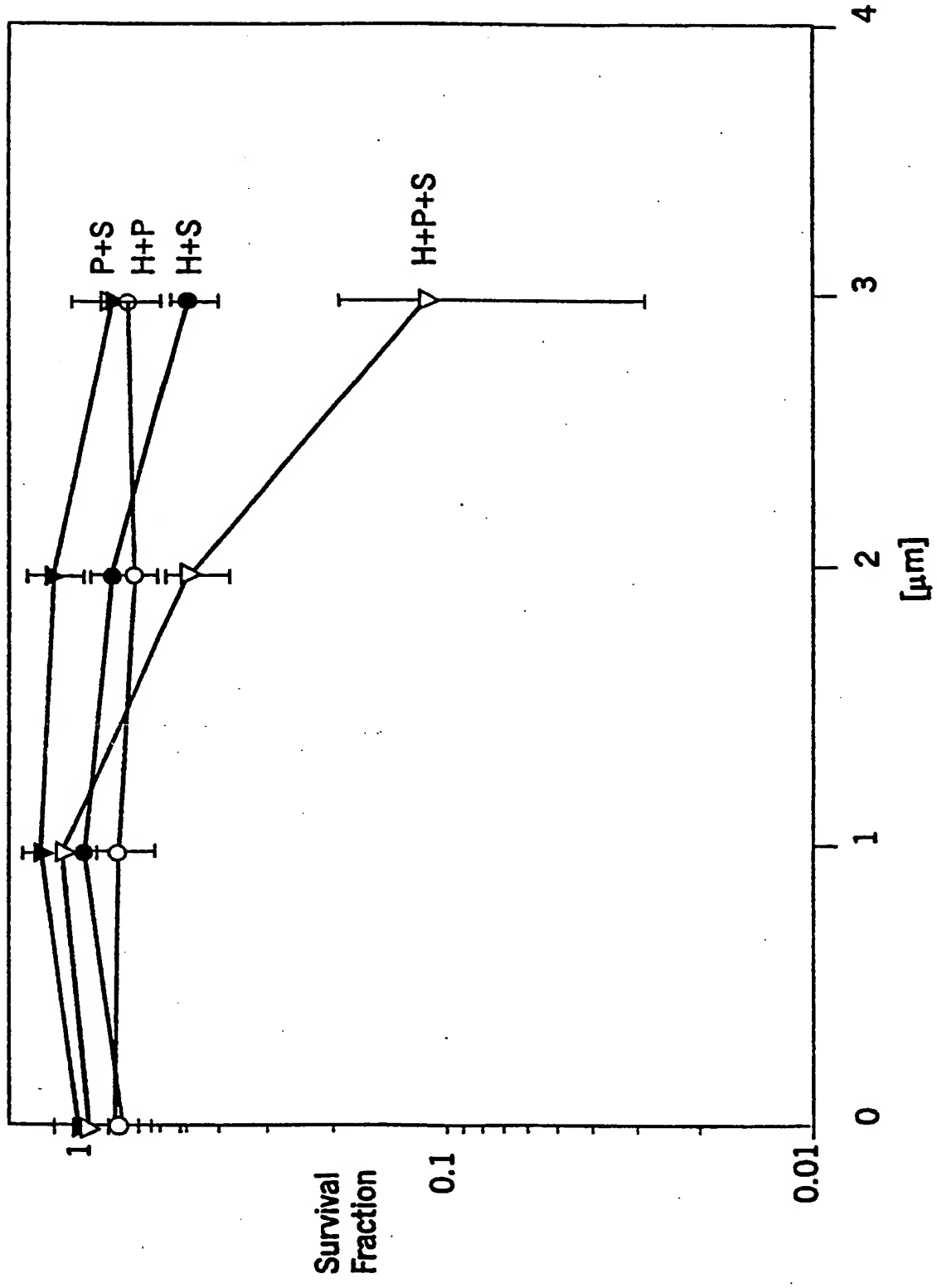


FIG. 7

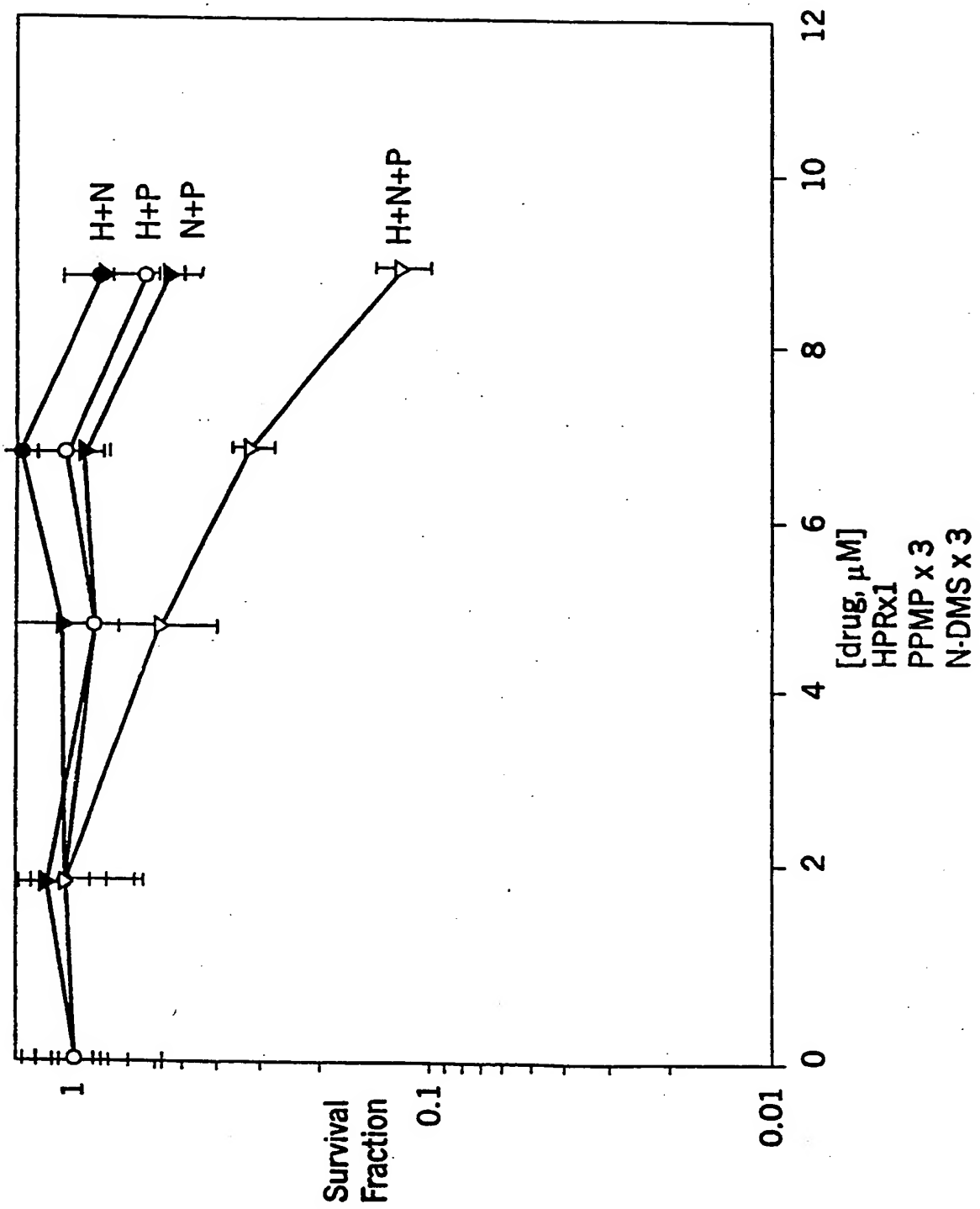

H+N
H+P
N+P
H+N+P
Survival Fraction
1
0.1
0.01
0
2
4
6
8
10
12
[drug, μM]
HPRx1
PPMP x 3
N-DMS x 3

FIG. 8

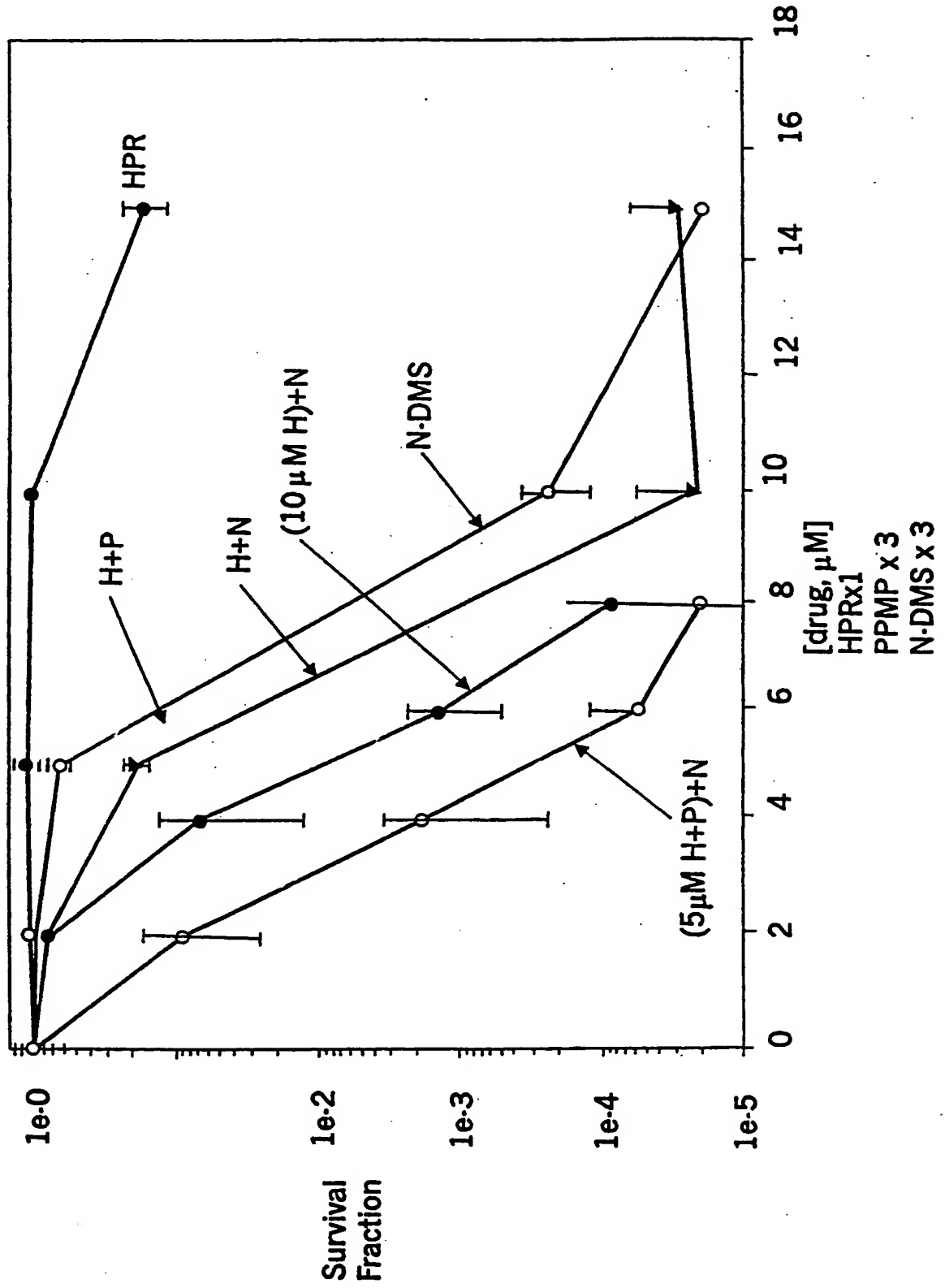

HPR
H+P
H+N
(10 μM H)+N
N-DMS
(5μM H+P)+N
Survival Fraction
1e-0
1e-2
1e-3
1e-4
1e-5
0
2
4
6
8
10
12
14
16
18
[drug, μM]
HPRx1
PPMP x 3
N-DMS x 3

FIG. 9

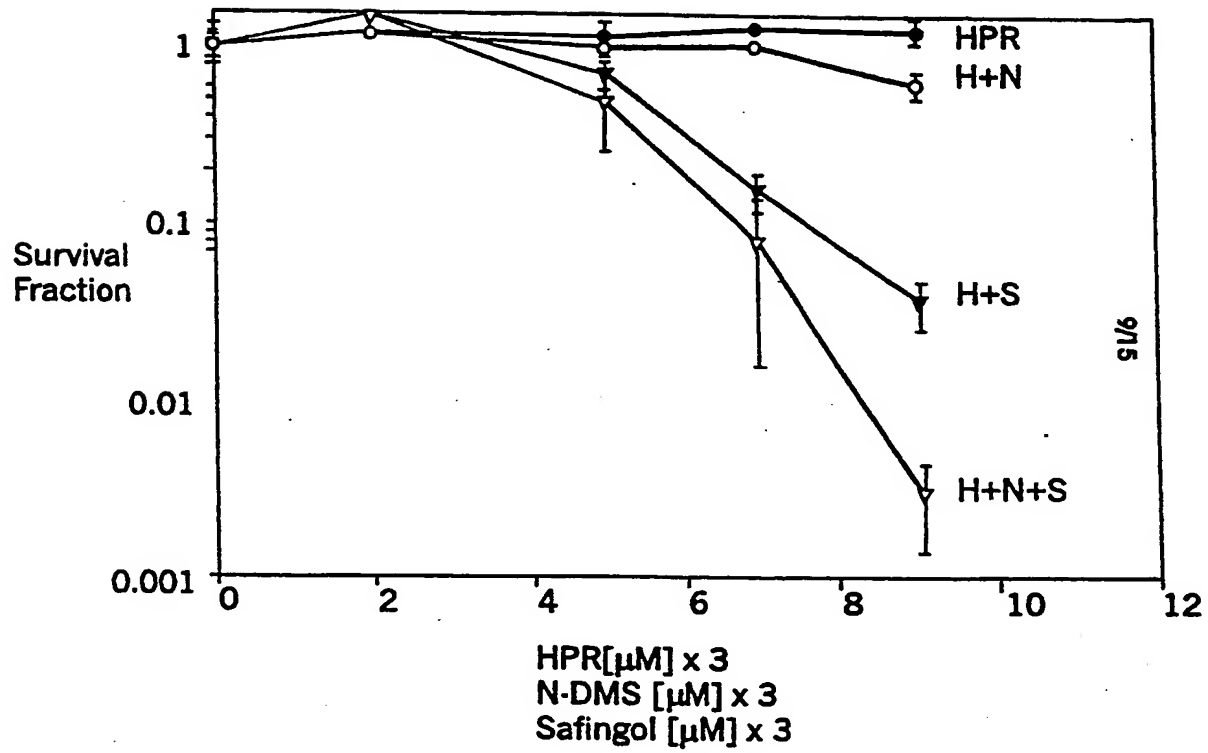

1
0.1
0.01
0.001
Survival Fraction
0
2
4
6
8
10
12
HPR
H+N
H+S
H+N+S
9/15
HPR[μM] x 3
N-DMS [μM] x 3
Safingol [μM] x 3

FIG. 10

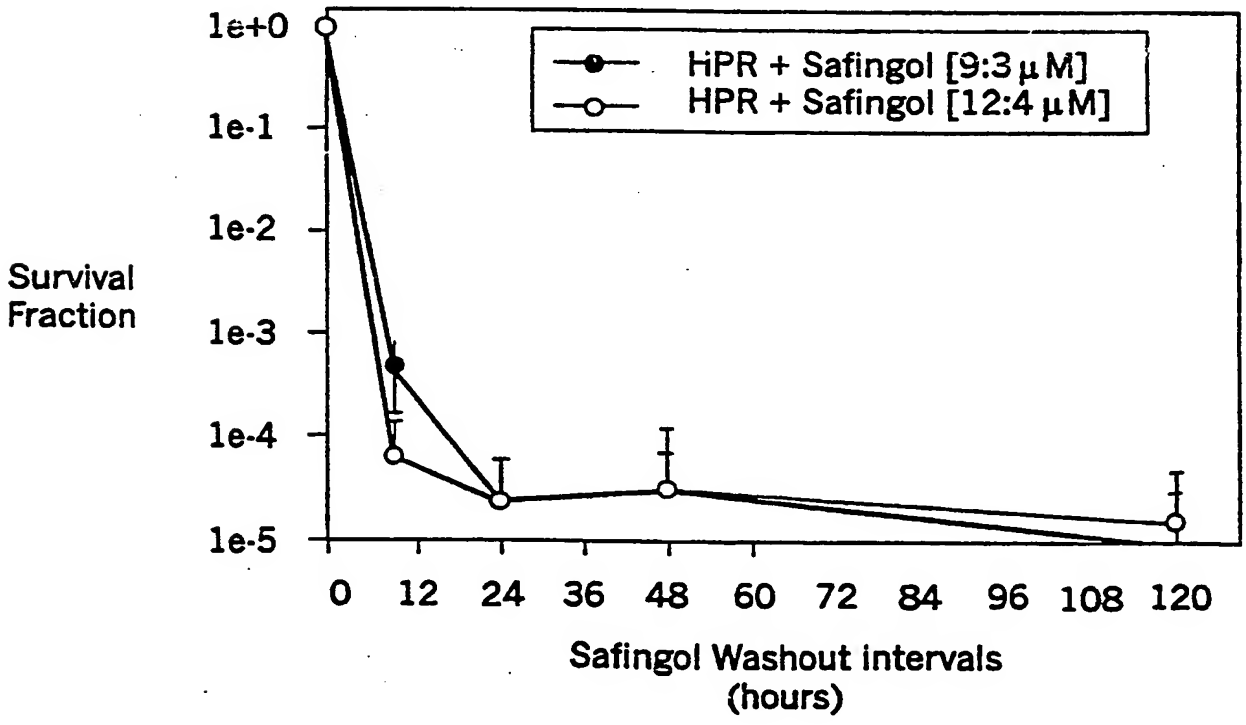

1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Survival Fraction
HPR + Safingol [9:3 μM]
HPR + Safingol [12:4 μM]
0
12
24
36
48
60
72
84
96
108
120
Safingol Washout intervals (hours)

FIG. 11

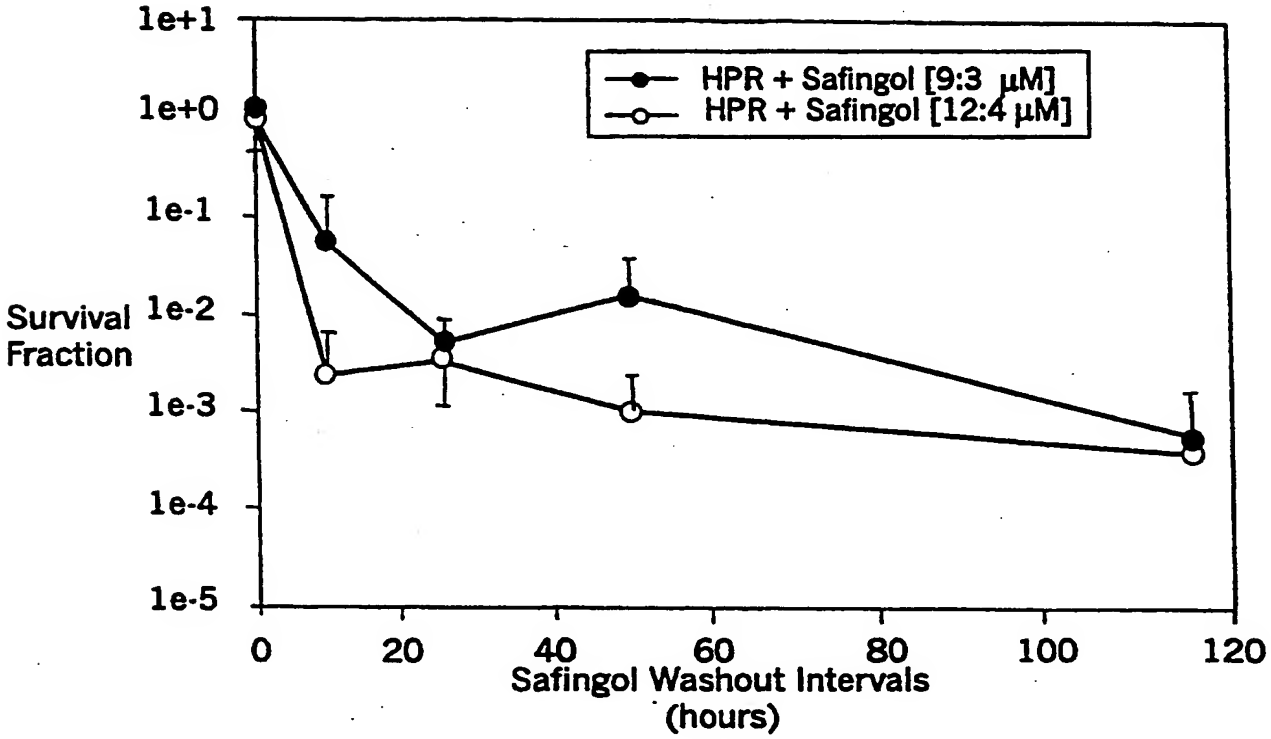

1e+1
1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Survival Fraction
HPR + Safingol [9:3 µM]
HPR + Safingol [12:4 µM]
0
20
40
60
80
100
120
Safingol Washout Intervals (hours)

FIG. 12

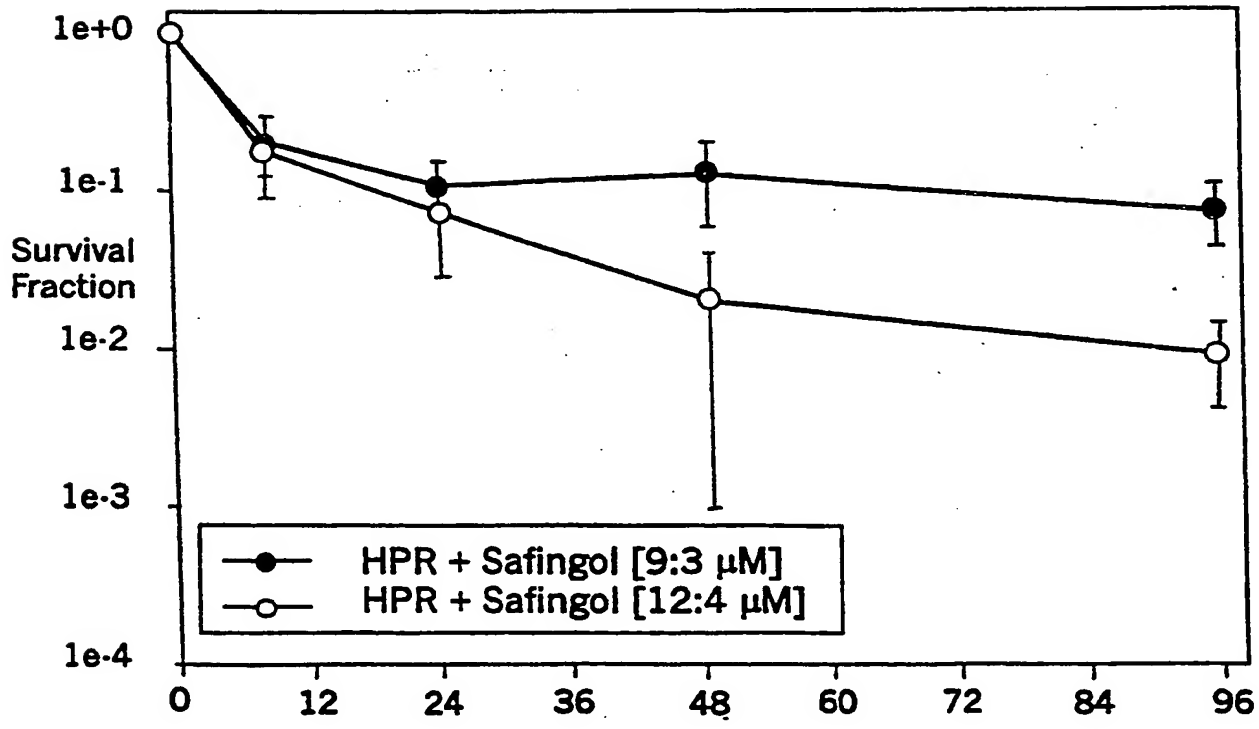

1e+0
1e-1
1e-2
1e-3
1e-4
Survival Fraction
HPR + Safingol [9:3 µM]
HPR + Safingol [12:4 µM]
0
12
24
36
48
60
72
84
96

FIG. 13

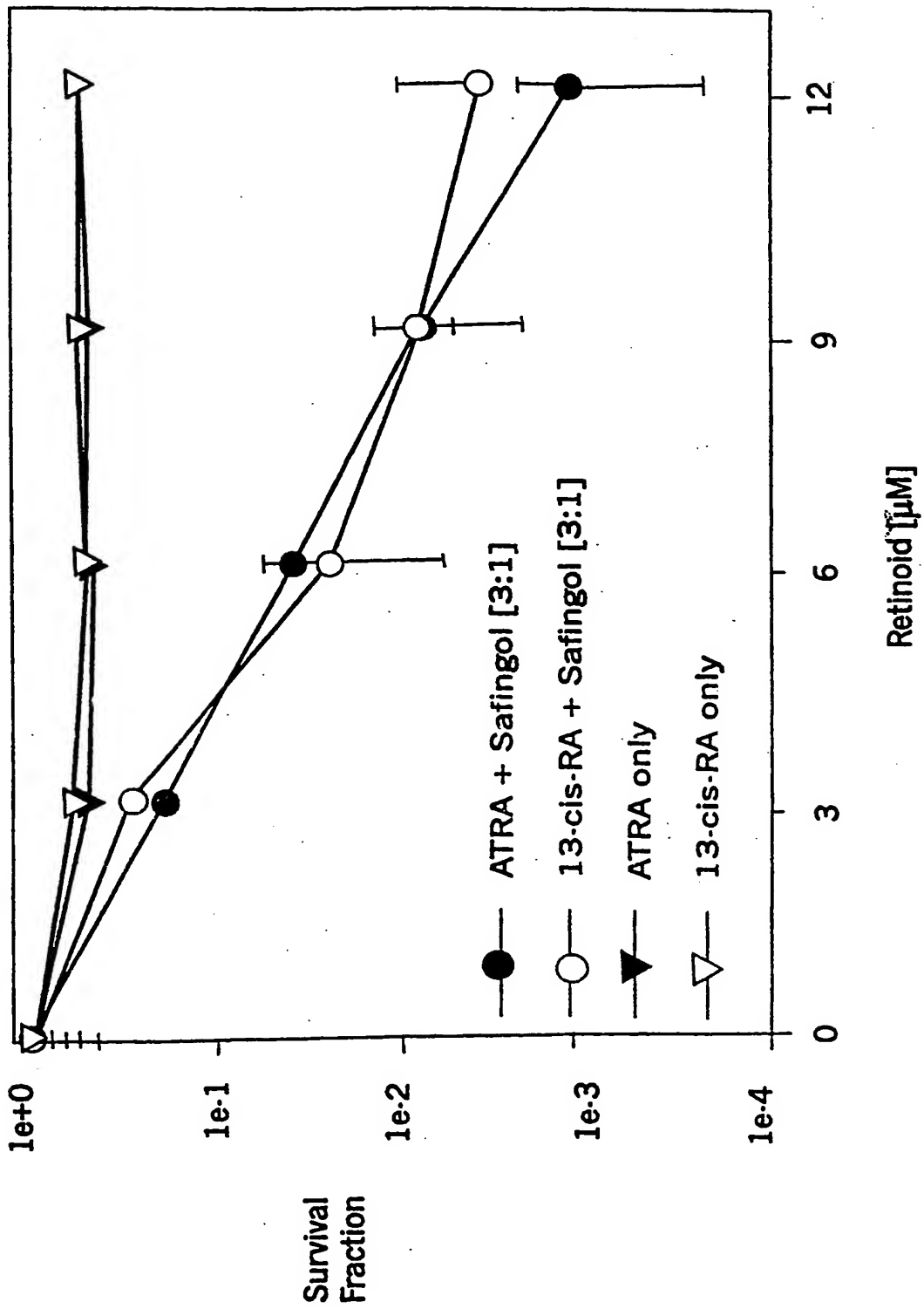

Survival Fraction
1e+0
1e-1
1e-2
1e-3
1e-4
0
3
6
9
12
Retinoid [μM]
ATRA + Safingol [3:1]
13-cis-RA + Safingol [3:1]
ATRA only
13-cis-RA only

FIG. 14

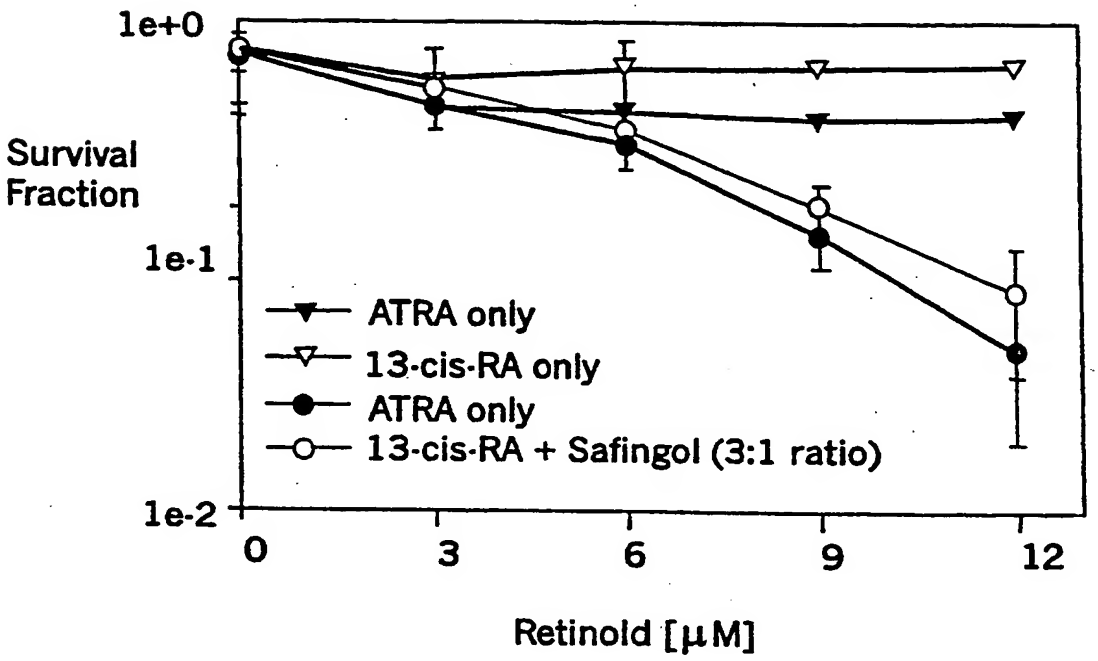

1e+0
1e-1
1e-2
Survival Fraction
ATRA only
13-cis-RA only
ATRA only
13-cis-RA + Safingol (3:1 ratio)
0
3
6
9
12
Retinoid [μM]

FIG. 15

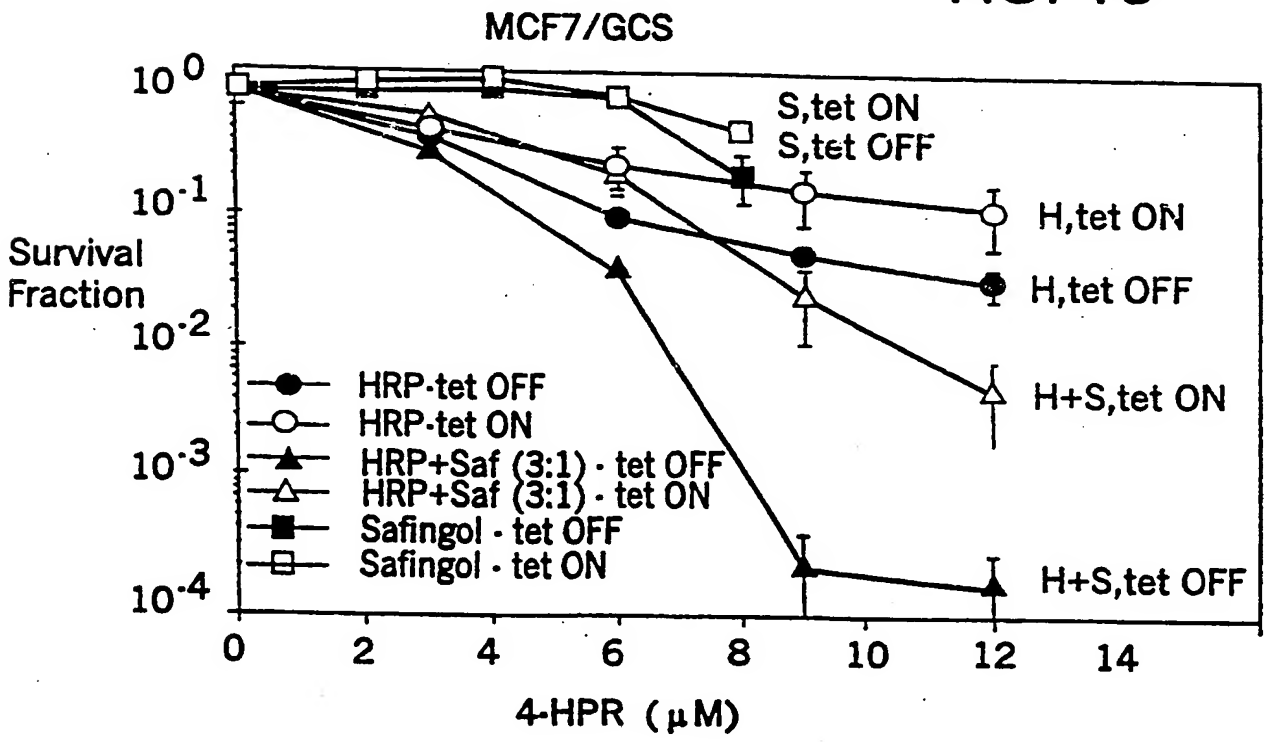

MCF7/GCS
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Survival Fraction
S,tet ON
S,tet OFF
H,tet ON
H,tet OFF
H+S,tet ON
H+S,tet OFF
HRP-tet OFF
HRP-tet ON
HRP+Saf (3:1) - tet OFF
HRP+Saf (3:1) - tet ON
Safingol - tet OFF
Safingol - tet ON
0
2
4
6
8
10
12
14
4-HPR (μM)

FIG. 16

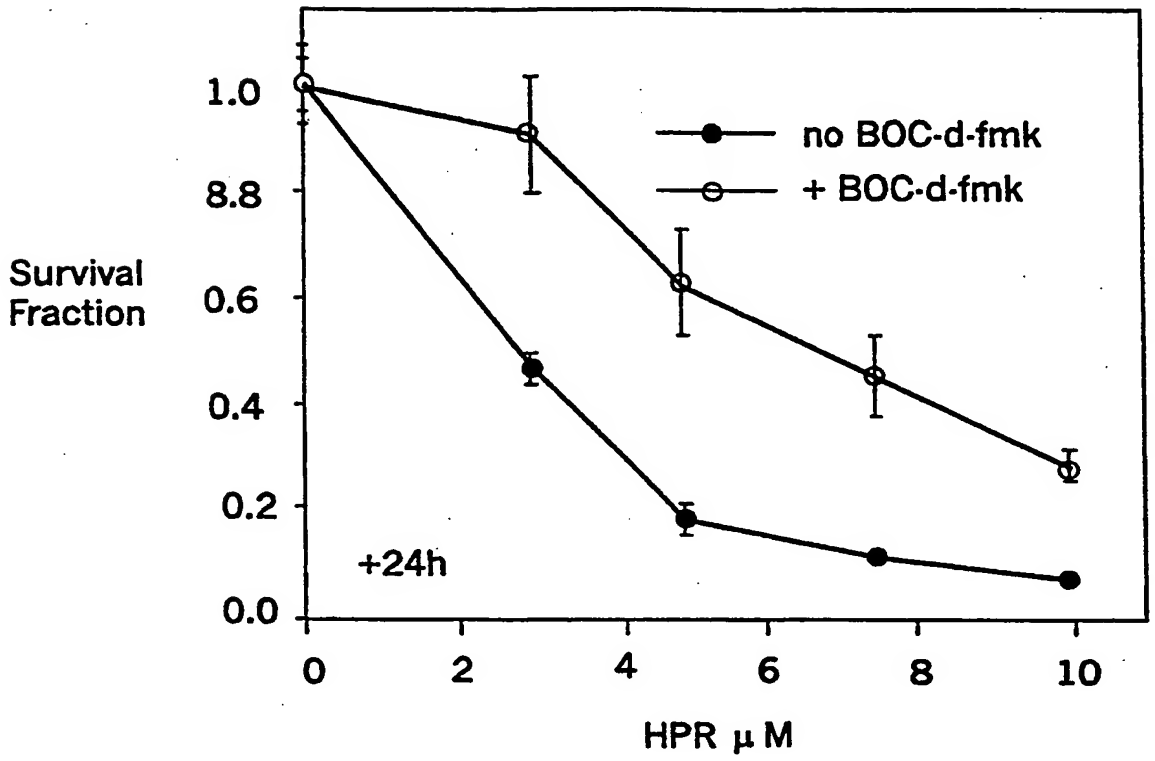

Survival Fraction
1.0
8.8
0.6
0.4
0.2
0.0
no BOC-d-fmk
+ BOC-d-fmk
+24h
0
2
4
6
8
10
HPR μ M

FIG. 17

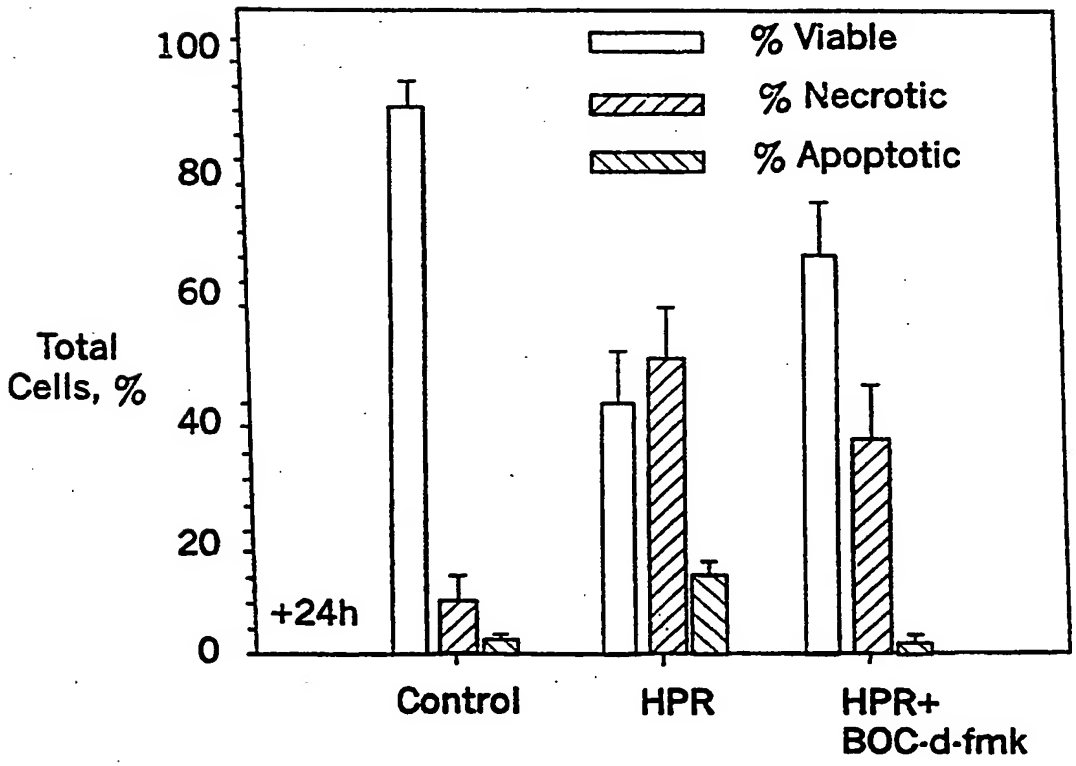

Total Cells, %
100
80
60
40
20
0
% Viable
% Necrotic
% Apoptotic
+24h
Control
HPR
HPR+ BOC-d-fmk

FIG. 18A

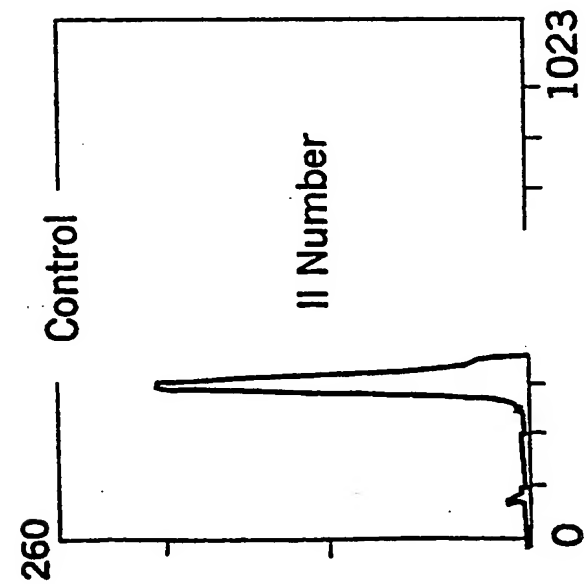


FIG. 18B

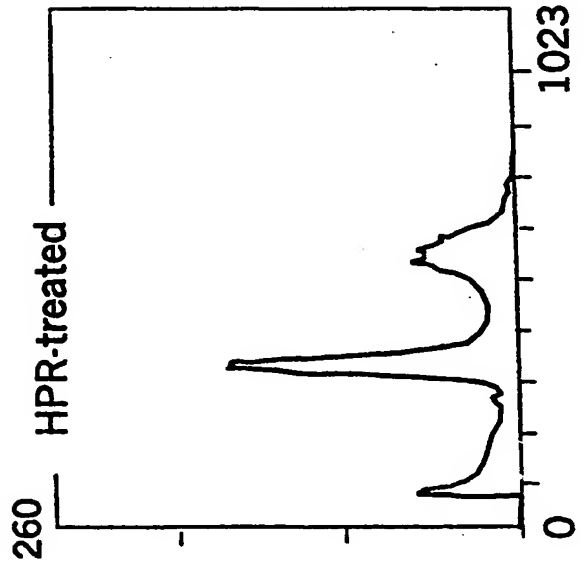


FIG. 18C

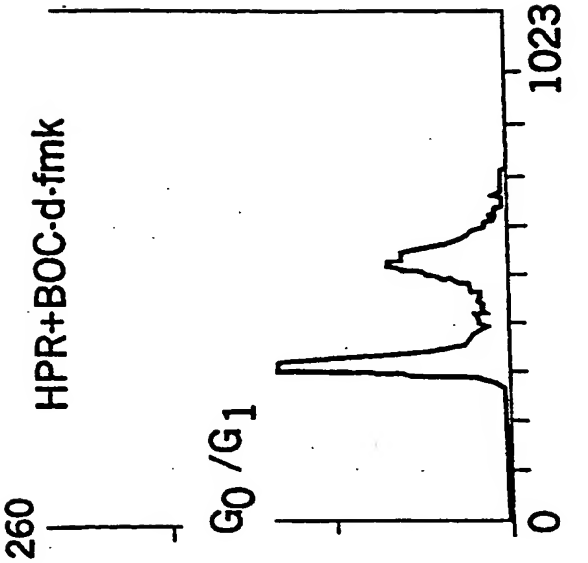
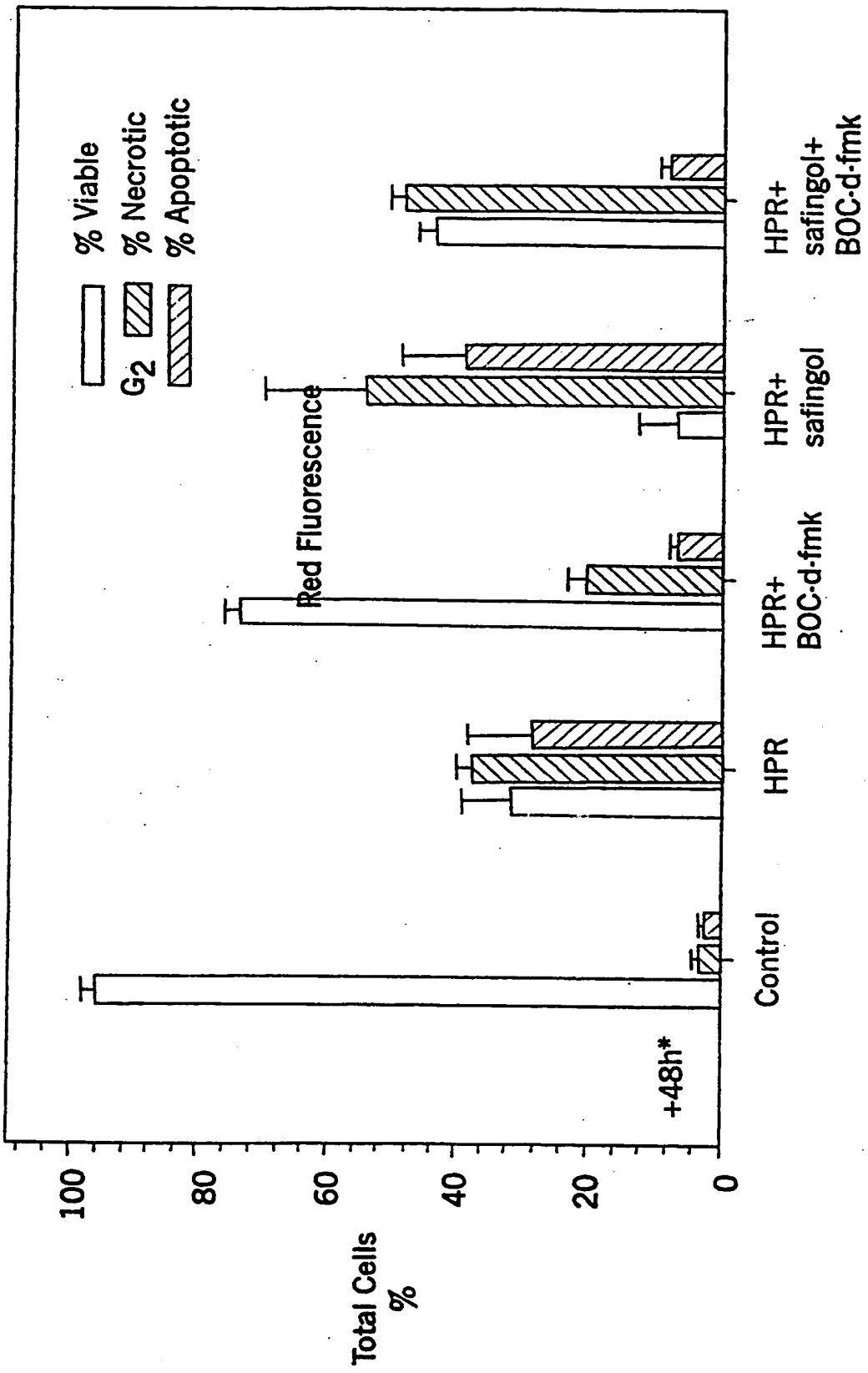


FIG. 19


% Viable
% Necrotic
% Apoptotic
G2
Red Fluorescence
Total Cells %
100
80
60
40
20
0
+48h*
Control
HPR
HPR+ BOC-d-fmk
HPR+ safingol
HPR+ safingol+ BOC-d-fmk

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14591

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70, 31/165, 31/13, 31/66, 31/20, 31/07.
US CL :514/617, 669, 24, 25, 114, 559, 725.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/617, 669, 24, 25, 114, 559, 725.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: cancer, dihydrosphingosine, fenretinide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,816,450 A (BELL et al.) 28 March 1989, column 1, line 66 to column 2, line 20	1-33
Y	US 5,464,870 A (VERONESI et al.) 07 November 1995, column 3, lines 15 to 34.	1-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be consid ··d novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 1999	22 OCT 1999

Name and mailing address of the ISA/US	Authorized officer
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